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Dual-function protein in plant defence: seed lectin from *Dolichos biflorus* (horse gram) exhibits lipoxygenase activity

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Plant–pathogen interactions play a vital role in developing resistance to pests. *Dolichos biflorus* (horse gram), a leguminous pulse crop of the subtropics, exhibits amazing defence against attack by pests/pathogens. Investigations to locate the possible source of the indomitable pest resistance of *D. biflorus*, which is the richest source of LOX (lipoxygenase) activity, have led to a molecule that exhibits LOX-like functions. The LOX-like activity associated with the molecule, identified by its structure and stability to be a tetrameric lectin, was found to be unusual. The evidence for the lectin protein with LOX activity has come from (i) MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS, (ii) N-terminal sequencing, (iii) partial sequencing of the tryptic fragments of the protein, (iv) amino acid composition, and (v) the presence of an Mn^{2+} ion. A hydrophobic binding site of the tetrameric lectin, along with the presence of an Mn^{2+} ion, accounts for the observed LOX like activity. This is the first ever report of a protein exhibiting both haemagglutination and LOX-like activity. The two activities are associated with separate loci on the same protein. LOX activity associated with this molecule adds a new dimension to our understanding of lectin functions. This observation has wide implications for the understanding of plant defence mechanisms against pests and the cellular complexity in plant–pathogen interactions that may lead to the design of transgenics with potential to impart pest resistance to other crops.

Key words: *Dolichos biflorus* (horse gram), dual-function protein, lipoxygenase activity, plant defence, plant–pathogen interaction, seed lectin.

INTRODUCTION

The population of the subtropics, being predominantly vegetarian, looks to legumes such as pigeon pea (*Cajanus cajan* or *Cajanus indicus*), chickpea (*Cicer arietinum*), mung bean (*Vigna radiata*) and horse gram (*Dolichos biflorus*) for the fulfilment of its need of dietary proteins. Insect pests are a major menace that destabilizes agricultural productivity. A variety of insect pests, ranging from leptidopterans to orthopterans, damage crops and stored seeds [1]. Protease inhibitors and lectins are part of the defence mechanisms in plants which get activated in response to insect attacks [2].

LOX (lipoxygenase or linoleate:oxygen oxidoreductase; EC 1.13.11.12) catalyses the regio/stereo-specific dioxygenation of PUFAs (polyunsaturated fatty acids) containing the *cis*,*cis*-1,4-pentadiene system [3,4]. Plant LOXs are versatile multifunctional enzymes that catalyse (i) dioxygenation of lipid substrates (dioxygenase reaction), (ii) secondary conversion of hydroperoxy lipids (hydroperoxidase reaction), and (iii) formulation of epoxy leukotrienes (leukotriene synthase reaction). Linoleic acid is oxygenated at either C-9 or C-13 of the hydrocarbon backbone of the fatty acid, leading to two groups of compounds, the (9*S*)-hydroperoxy and the (13*S*)-hydroperoxy derivatives of PUFAs [5].

The products of the linoleic acid pathway mediate the wound-induced resistance in plants. The products from 13-hydroperoxylinolenic acid can be elaborated further by enzymatic cyclization, reduction and β-oxidation to produce jasmonic acid. LOX proteins contribute to plant growth and development, maturation, senescence and trigger metabolic responses to pathogen attack [6,7]. Animal and plant cells, based on LOX activation, share a similar signal transduction pathway capable of triggering apoptosis after oxidative stress [8].

LOXs contain ferrous iron, which is oxidized into the ferric state. The ferric form of LOXs is catalytically active and catalyses the stereospecific abstraction of one hydrogen from the bis-allylic methylene group of the (1*Z*,4*Z*)-pentadiene structure of the substrate as the initial step [9]. Mammalian and plant LOXs catalyse production of hydroperoxides that have the *S* absolute configuration. Starfish, sea urchins and the coral *Plexaura homomalla* express LOXs which catalyse the formation of *R* hydroperoxides [10]. LOX, purified from the fungus *Gaeumannomyces graminis*, contained 0.5–1.0 atom of manganese per molecule [11].

Lectins are proteins that have affinity for specific carbohydrate moieties. They bind to glycoproteins in the peritrophic matrix lining the insect midgut and disrupt digestive processes and nutrient assimilation [1]. Lectins are particularly abundant in the seeds of legumes. They constitute up to 10 % of the soluble protein in the seed extracts [12]. Lectins are used as a model system for studying protein–carbohydrate interactions. The legume lectins, despite the high sequential and structural similarity of their subunits, show a remarkable range of sugar specificities [13].

The number of proteins present in any cell is limited, yet the functions performed by them are many. The protein surface can provide multiple active sites of various catalytic potentials. There are many proteins that are reported to have multiple functions associated with them, described as moonlighting proteins [14]. The multiple functions associated with these moonlighting proteins add new dimensions to cellular complexity. The moonlighting proteins may provide one way of co-ordinating cellular activities.

As part of the ongoing study to understand the structure and stability of LOXs from legumes [15], we report the purification of

Abbreviations used: ANS, 8-anilinonaphthalene-1-sulphonate; ETYA, eicosa-5,8,11,14-tetraynoic acid; HOD, hydroxyoctadecadienoic acid; HPOD, hydroperoxyoctadecadienoic acid; LOX, lipoxygenase; MALDI, matrix-assisted laser-desorption ionization; NDGA, nordihydroguaiaretic acid; PUFA, polyunsaturated fatty acid; RP, reverse phase; SP, straight phase; TFA, trifluoroacetic acid; TOF, time-of-flight; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane.

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a dual-function protein from *D. biflorus* seeds. This molecule exhibits both lectin and LOX-like activity. From the amino acid sequence and amino acid composition data, it was identified to be a tetrameric lectin. This is the first report of a lectin having LOX activity, which may help explain the indomitable pest resistance enjoyed by this unusual legume.

MATERIALS AND METHODS

D. biflorus seeds, Nagamangala Pac 9 strain, were procured from the University of Agricultural Sciences, Bangalore, India. *D. biflorus* seeds were finely ground and defatted with hexane, dried and stored at 4 °C until use.

Linoleic acid (> 99 %) and linolenic acid (> 99 %) were obtained from Nuchek Prep. Arachidonic acid, DEAE-Sephacel, Sephacryl S-200, Tween 20, protein molecular-mass markers, protease inhibitor cocktail, urea, NDGA (nordihydroguaiaretic acid), 4-nitrocatechol, *D. biflorus* lectin, nitrocellulose membrane, β-carotene, Ponceau stain, ETYA (eicosa-5,8,11,14-tetraynoic acid), *N*-acetylgalactosamine, TFA (trifluoroacetic acid), Freund's complete adjuvant, Freund's incomplete adjuvant, goat anti-rabbit IgG conjugate, ANS (8-anilinonaphthalene-1-sulphonate), BSTFA [bis(trimethylsilyl)trifluoroacetamide], adenine, kinetin, Caps and Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulphonamide) were from Sigma Chemical Co. Solvents used were of HPLC grade. All other chemicals were of analytical grade.

Protein purification

Defatted *D. biflorus* flour (2.5 g) was extracted with 0.1 M sodium phosphate buffer, pH 6.0, containing protease inhibitor cocktail and 0.01 % sodium azide for 2 h with gentle stirring at 4 °C. The slurry was centrifuged at 15000 ***g*** for 30 min at 4 °C to recover the supernatant. Protein in the supernatant was precipitated with ammonium sulphate (0–70 % saturation) to recover the pellet with LOX activity. The pellet was dissolved in 25 ml of 20 mM sodium phosphate buffer (pH 6.0) and dialysed against the same buffer containing 5 % glycerol and 0.01 % sodium azide (buffer A) (6 × 1000 ml) before fractionation on a Sephacryl S-200 column (2.7 cm × 120 cm, 700 ml) pre-equilibrated using buffer A at a flow rate of 20 ml/h. Activity was recovered in three peaks with the highest specific activity (94 units/mg) in the second peak (35 % of total activity loaded). This peak was pooled, dialysed against buffer A containing 0.05 M NaCl and fractionated on a DEAE-Sephacel column (2.1 cm × 7 cm, 25 ml) equilibrated with the same buffer using a linear gradient of 0.05–0.3 M NaCl at a flow rate of 10 ml/h. The active fractions were pooled, and the homogeneity was evaluated by SDS/12 % PAGE and RP (reverse-phase)-HPLC. The protein was stored at − 20 °C following flushing with nitrogen.

Purification of seed lectin from *D. biflorus*

The lectin from *D. biflorus* seeds was purified as reported previously [16], with slight modifications. Instead of water, the purification was carried out in 0.1 M sodium phosphate buffer, pH 6.0, at 4 °C.

Electrophoresis

SDS/PAGE, native PAGE and activity-staining-SDS/PAGE were carried out using 12 % polyacrylamide gels [17]. Native PAGE was carried out using 12 % gels without SDS or 2-mercaptoethanol. The molarity of the electrophoretic buffer used (for native gels) was 50 mM Tris/HCl and 284 mM glycine. The gels were over-run for 0.5 h (in the case of native PAGE) and were stained with Coomassie Brilliant Blue R-250.

Activity staining was used to detect LOX in polyacrylamide gels under native conditions. The gels were run at 4 °C as reported previously [18]. Gels were incubated in 0.1 M sodium phosphate buffer, pH 6.0, containing linoleic acid (50 mg solubilized in ethanol, made up to 100 ml with 0.1 M sodium phosphate buffer, pH 6.0) for 6 h at 4 °C and were stained for activity.

LOX assay

LOX activity was monitored by UV analysis, following the method of Shimizu et al. [19], by monitoring the increase in absorbance at 234 nm due to the formation of conjugated diene fatty acid hydroperoxide. One unit of enzyme activity was defined as the amount of enzyme required to form 1 μmol of product/min at 25 °C under the assay conditions. The standard assay mixture consisted of 150 μM linoleic acid in 0.1 M sodium acetate buffer (pH 4.5) containing 2 nM Tween 20. Fresh stock solutions (10 mM) of the desired fatty acid in absolute ethanol were prepared for enzyme assay. All buffers were freshly prepared. The reaction was initiated by the addition of enzyme to substrate in a total volume of 3 ml at 25 °C and was monitored for 3 min. To the reference cuvette, buffer with alcohol was added instead of enzyme. The rate was estimated from the linear part of the curve. Kinetics constants were determined at 25 °C in triplicate for $C_{18:2,n-6}$, $C_{18:3,n-3}$ and $C_{20:4,n-6}$. Protein concentrations were determined following the method of Lowry et al. [20], using BSA as standard. A molar absorption coefficient of 25000 $M^{-1} \cdot cm^{-1}$ was used for determining the concentration of the hydroperoxides.

Haemagglutination assay

Haemagglutination assay was carried out by serial dilution of the lectin in PBS (0.01 M sodium phosphate and 0.15 M sodium chloride, pH 7.0) using 2 % trypsinized A^+ human blood as reported previously [21]. Haemagglutination was observed visually, with 1 HU defined as the amount of protein required to cause visible agglutination using human A^+ erythrocytes. The specific activity is given as units/mg of protein.

Determination of homogeneity and molecular mass of purified protein by HPLC

Homogeneity was also ascertained by RP-HPLC, using a Shim Pak C_{18} (10 μ, 4.6 mm × 250 mm) column with solvent A (0.1 % TFA in water) and solvent B (100 % acetonitrile containing 0.1 % TFA). Protein (20 μg) was injected at a flow rate of 1 ml/min. The column was washed with solvent A for 5 min and brought to 50 % acetonitrile in 10 min. The bound protein was eluted with a linear gradient of acetonitrile (50–75 %) over a period of 45 min. Detection was monitored at 280 nm using Waters® 2996 photodiode array detector.

The molecular mass of purified LOX was determined by gel filtration on HPLC using a TSK G-2000 SW_{XL} (7.8 mm × 300 mm, 5 μm) column, equilibrated in 20 mM sodium phosphate buffer, pH 6.0, containing 0.3 M NaCl. Elution was carried out at a flow rate of 0.2 ml/min for 45 min; protein was detected at 280 nm. The column was calibrated using standard proteins [alcohol dehydrogenase, 150000 Da; soya bean (*Glycine max*) LOX1, 94000 Da; BSA, 68000 Da; carbonic anhydrase, 29000 Da].

Fractionation of subunits

Fractionation of subunits was carried out by ion-exchange chromatography on a DEAE-cellulose column (2.1 cm × 7 cm) equilibrated with 8.0 M urea in 0.04 M Tris/HCl buffer, pH 7.3,

at 25 °C. Sample (15 mg) was dialysed against 10.0 M urea in 0.04 M Tris/HCl, pH 7.3, and was applied to the column [22]. The subunits, recovered in two separate peaks, were pooled and dialysed extensively against 0.04 M Tris/HCl, pH 7.3, containing 0.02 % sodium azide at 4 °C. LOX activity of the separated subunits was checked.

MS

The subunits of *D. biflorus* LOX protein were analysed by MALDI (matrix-assisted laser-desorption ionization)–TOF (time-of-flight) MS after gel filtration on HPLC (as described above). The peak fraction was collected and analysed. MALDI MS analyses were performed on a Bruker Daltonics Ultraflex MALDI–TOF/TOF system (Bruker-Daltonics) in the reflection positive-ion mode, equipped with a nitrogen laser of 337 nm. The samples were prepared by mixing equal volumes of sample prepared in water/TFA (100:0.1, v/v) and saturated α-cyano-4-hydroxycinnamic acid matrix (Sigma–Aldrich) prepared separately in acetonitrile/water/TFA (80:20:0.1, by vol.). The amount of protein loaded on the probe slide was approx. 10 pmol. The samples were then dried at 25 °C under atmospheric pressure. Data were collected between 0 and 100 000 Da.

Substrate affinity

Substrate affinity of the purified *D. biflorus* LOX protein towards different fatty acids was carried out at the optimum pH of 4.5 in 0.1 M sodium acetate buffer at 25 °C. The protein was incubated with different concentrations (10–300 μM) of linoleic acid, linolenic acid and arachidonic acid, and the assay was performed as given above. The K_m and V_{max} were estimated from a double-reciprocal plot of substrate concentration against reaction rate.

Co-oxidation of β-carotene

Bleaching of the pigment, β-carotene, by LOX was monitored spectrophotometrically at 460 nm [23]. Linoleic acid solution (10 mM) was prepared following the method of [24] in 50 mM sodium phosphate buffer, pH 6.0. The co-oxidation reaction was carried out by incubating substrate with different amounts of LOX for 10 min at 20 °C.

Inhibition of LOX and lectin activities

LOX activity was carried out in the presence of various concentrations of (i) 4-nitrocatechol (0–600 μM), (ii) ETYA (0–200 μM), and (iii) NDGA (0–500 μM) after pre-incubation of the protein for 5 min in the presence of the test inhibitor at 25 °C. Stock solutions of NDGA and ETYA were prepared in absolute ethanol while 4-nitrocatechol was prepared in 50 mM sodium phosphate buffer, pH 6.0. The LOX assay was performed as described above.

Inhibition of LOX activity by adenine (0–500 μM), kinetin (0–500 μM) and ANS (0–100 μM) was studied by incubating the lectin in the presence of the test inhibitor for 5 min and assaying the LOX activity as described above.

The *D. biflorus* LOX protein was incubated with *N*-acetylgalactosamine (0–1 mM) for 5 min at 25 °C. The lectin assay was carried out as described above.

Detection of LOX and lectin activity loci

To confirm the distinctness of the LOX locus of the protein from its sugar binding (lectin) locus, *N*-acetylgalactosamine (500 μM) in PBS was added to the protein, and the assay for LOX was carried out using appropriate blanks. Similarly, haemagglutination activity was assayed in the presence of the LOX inhibitors NDGA (500 μM) and ETYA (300 μM).

Isolation, identification and characterization of LOX products

The products of the LOX reaction with linoleic acid were isolated as described previously [25]. Diazomethane derivatives were prepared, redissolved in methanol and identified by SP (straight-phase)-HPLC. SP-HPLC was carried out using a Waters® Chromatograph equipped with Sorbax-Sil column (4.6 mm × 250 mm, 5 μm). Positional isomers of the linoleic acid oxygenation products were separated and detected at 234 nm using isocratic elution with the n-hexane/isopropyl alcohol/ethanoic (acetic) acid (100:2:0.1, by vol.) solvent system. The products of potato (*Solanum tuberosum*) LOX (purified following the method in [26]) and soya bean LOX (purified following the method in [27]), using alcohol-solubilized fatty acids at pH 5.5 and 9.0, were used as standards.

Enantiomer composition of the hydroxy fatty acid was analysed by chiral-phase HPLC on a Bakerbond chiral-phase column (dinitrobenzoyl phenylglycine coupled ionically over aminopropyl residues on silica gel as chiral phase; JT Baker Products, 250 mm × 4 mm, 5 μm). The column was eluted with 0.5 % propan-2-ol in n-hexane [28] at a flow rate of 0.8 ml/min. Products of pea (*Pisum sativum*) seed LOX1 [~23 % L-(*S*)-13-hydroperoxide and ~16 % D-(*R*)-13-hydroperoxide of products produced] [28,29] and soya bean LOX1 [predominantly L-(*S*)-13-hydroperoxide, ~98%] were used as the standards [28].

GC-MS analyses

GC-MS analysis of the products of the LOX reaction with linoleic acid was performed using a GC17A QP5000 Mass Spectrometer (Shimadzu). Capillary GC (Shimadzu) was carried out on a non-polar column (30 m DB-1, J&W Scientific; film, 0.25 μm; diameter 0.25 mm) following the method in [30].

Amino acid composition

The peak fraction of purified *D. biflorus* LOX protein was analysed directly for total amino acids. The protein was blotted on to a PVDF membrane after SDS/PAGE. Electrophoretic transfer (Mini Trans-Blot®; Bio-Rad) was in 10 mM Caps, pH 11.0, with 10 % (v/v) methanol (100 V for 4 h at 25 °C) and the membranes were stained with Ponceau stain. The excised protein band was subjected to total amino acid analysis. Amino acid analysis was performed by precolumn derivatization using phenylisothiocyanate. The phenylthiocarbamyl amino acids were analysed by RP-HPLC [31] using a Waters® Associates HPLC and an application-specific Pico-Tag™ amino acid analysis column (15 cm × 3.9 mm) with a binary gradient system. The phenylthiocarbamyl amino acids were detected at 254 nm.

Amino acid sequence of the protein

The peak fraction of purified *D. biflorus* LOX protein and fractionated subunits were analysed directly for N-terminal amino acid sequence on an Applied Biosystems Procise® 4.0 instrument. The protein/subunit was blotted on to a PVDF membrane as described for amino acid composition, and the excised band was subjected to N-terminal sequencing.

Internal peptide sequencing was carried out after tryptic digestion. The large peptide fragments obtained by limited digestion with Tos-Phe-CH_2Cl (tosylphenylalanylchloromethane, 'TPCK')-treated trypsin were separated by RP-HPLC using a Shim Pak C_{18} (10 μm, 4.6 mm × 250 mm) column with solvent A (0.1 % TFA in water) and solvent B (70 % acetonitrile containing 0.05 % TFA). The tryptic digest was injected at a flow rate of 0.8 ml/ min. The column was washed with solvent A for 5 min, and the bound protein was eluted by a linear gradient of acetonitrile

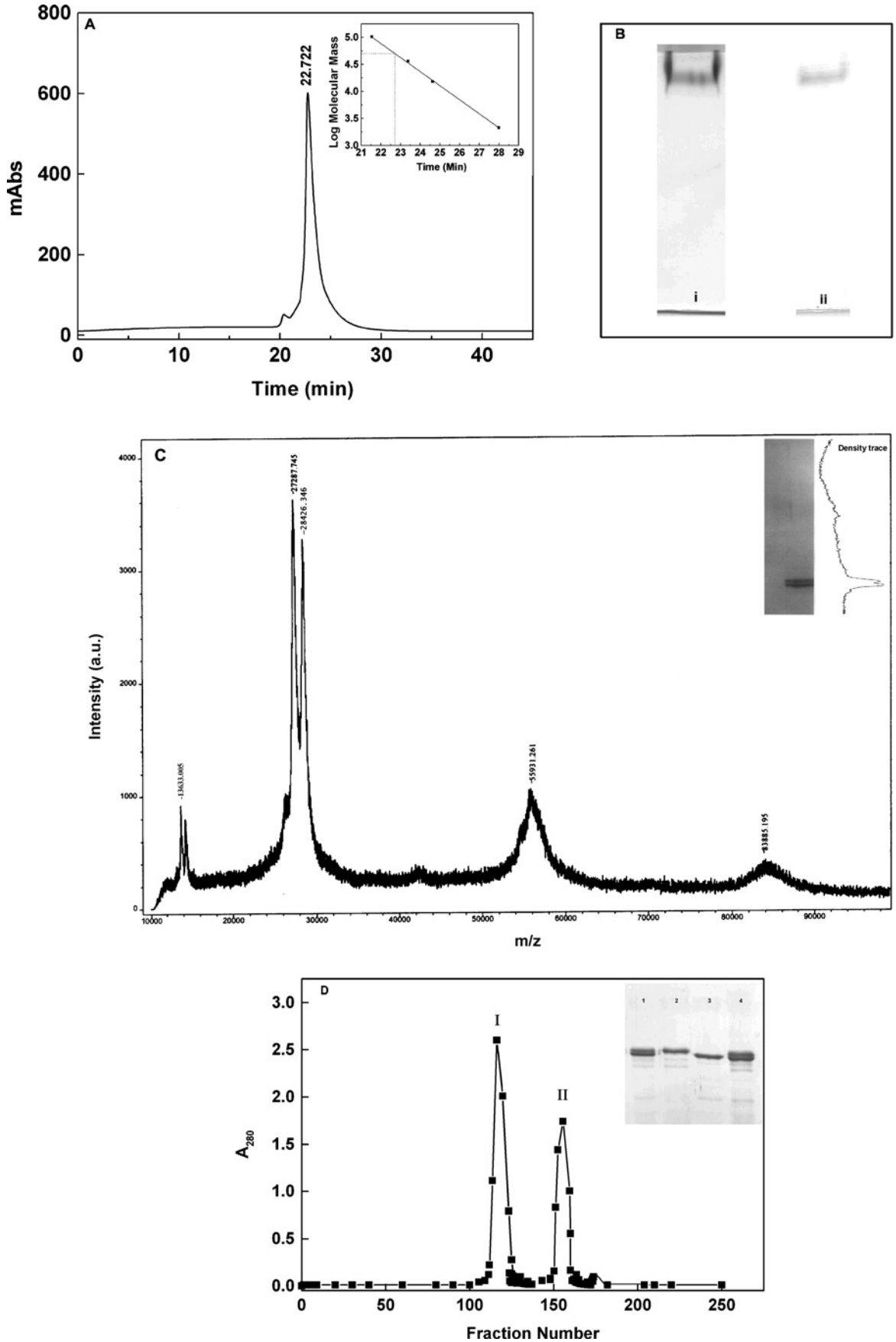

Figure 1 For legend see facing page

(0–70 %) over a period of 90 min. Detection was monitored at 280 nm using Waters® 2996 photodiode array detector.

Sequence alignment

The N-terminal sequence of 30 amino acids and two internal peptide sequences obtained by tryptic digestion were aligned with the *D. biflorus* lectin (PDB code 1LU1) using Clustal W version 1.82.

Spectroscopic measurements

Light-absorption spectra of *D. biflorus* LOX protein and its products were recorded at 25 °C on a Shimadzu UV-1601 double-beam spectrophotometer in the range 220–600 nm with a 10 mm pathlength cell. Samples were in 20 mM sodium phosphate buffer, pH 6.0.

Fluorescence measurements were carried out using a Shimadzu RF 5000 spectrofluorimeter at 25 °C. Protein concentrations of 50 μg/ml in 20 mM sodium phosphate buffer, pH 6.0, were used. The sample was excited at 280 nm (excitation maximum) and the emission was recorded in the range 300–400 nm using slit widths of 5 nm for both excitation and emission.

CD measurements were performed on a Jasco J-810 automatic recording spectropolarimeter at 25 °C. Far-UV CD spectra (260–200 nm) were recorded using a 1 mm pathlength cell, while near-UV CD spectra (320–240 nm) were recorded using a 10 mm pathlength cell. Protein concentrations of 0.42 mg/ml and 1–1.5 mg/ml in 0.02 M sodium phosphate buffer, pH 6.0, were used for far-UV and near-UV CD spectra respectively. An average of three scans was obtained for each spectrum at a scan speed of 10 nm/min. The mean residue ellipticity $[\theta]_{MRW}$ was calculated using a value of 115 as calculated from amino acid data.

Thermal transition curves were obtained from the data collected at 217 nm, in the temperature range 25–85 °C using a Peltier attachment (PMH 354WI), at a heating rate of 1 °C/ min. Samples were degassed to prevent formation of air bubbles at high temperatures.

Thermal inactivation studies

The loss of LOX activity as a function of temperature was monitored in 20 mM sodium phosphate buffer, pH 6.0. Enzyme samples of 100 μl were incubated for 30 min at different temperatures ranging from 4 to 95 °C. After cooling to 4 °C, the residual activity was measured at 25 °C by transferring an aliquot of 10 or 20 μl to the assay mixture. The midpoint of thermal inactivation, T_m, at which the activity was diminished by 50 %, was calculated from the plot of percentage residual activity against temperature. Activity of the unincubated enzyme was taken as 100 %.

Kinetics of thermal inactivation of LOX activity was studied at different temperatures in the range 78–95 °C. Enzyme in 20 mM sodium phosphate buffer, pH 6.0, was incubated in a water bath at the test temperature, and 10 or 20 μl aliquots were withdrawn at appropriate time intervals. The enzyme was immediately cooled to 4 °C in an ice bath, and the residual activity was measured at 25 °C, as described above. LOX activity at 4 °C was taken as 100 %. From a semilogarithmic plot of residual activity against time, the inactivation rate constant k_r was calculated. The temperature dependence of k_r was analysed from the Arrhenius plot to obtain the inactivation constants.

Production of polyclonal antibodies against soya bean LOX1

Albino rabbits were immunized with 300 μg of soya bean LOX1 dissolved in 0.5 ml of 20 mM sodium phosphate buffer, pH 6.8, mixed with equal volumes of Freund's complete adjuvant as the first injection [32]. Booster doses of 150 μg of soya bean LOX1 with Freund's incomplete adjuvant were administered weekly after resting the animals for 21 days. After each booster dose, blood was collected from the ear vein of the animal, allowed to clot at room temperature (25 °C) and was centrifuged to recover the serum. Cross reactivity with *D. biflorus* purified lectin, purified *D. biflorus* LOX protein and soya bean LOX1 were evaluated after transferring to nitrocellulose membrane.

RESULTS

Protein purification

A protein from *D. biflorus* seeds, which exhibited both LOX and haemagglutination activity, was purified for further studies to confirm its characteristics. The results of the purification are given in Supplementary Table 1 at http://www.BiochemJ.org/bj/395/bj3950629add.htm. LOX activity in the purified protein had a specific activity of 154 units/mg and a yield of 25 % from the crude extract. RP-HPLC experiments for homogeneity of the preparation revealed a single peak with a retention time of 37 min (results not shown). Size-exclusion chromatography was carried out using standard proteins as markers in the molecular mass range 14 000–150 000 Da. The molecular mass of the purified protein was determined to be ~110 000 Da (Figure 1, inset). The protein of the present study (Figure 1A) had the same retention time (i.e. it co-eluted) of 22.72 min as the commercial seed lectin (results not shown). The native PAGE profile of the purified protein is given in Figure 1(B) (lane i).

The LOX activity in the protein was detected by activity staining (Figure 1B, lane ii). A part of the band corresponding to LOX activity was excised from the gel and the protein was eluted. This was used to determine haemagglutination activity. The homogeneous preparation showed haemagglutination of 45 ± 5 units/mg of protein. The LOX/lectin ratio remained constant throughout the purification, especially in the later stages of purification (see Supplementary Table 1). The crude extract had a ratio of 4.8:1 (LOX/lectin). Among the three peaks obtained from the Sephacryl S-200 column, haemagglutination was associated only with the second peak. The LOX/lectin ratio was 2.93:1 and this remained unchanged after DEAE-Sephacel chromatography, establishing that the protein had both LOX and haemagglutination activity associated with it. Commercial lectin from *D. biflorus* had

Figure 1 Establishing the homogeneity of *D. biflorus* LOX protein

(**A**) Size-exclusion HPLC of protein from *D. biflorus* with LOX activity for homogeneity and determination of molecular mass, showing a single peak at 22.7 min. Inset, determination of molecular mass. Molecular-mass markers in the range 29 000–150 000 Da were run, and the molecular mass was determined. (**B**) (i) Native PAGE and (ii) activity staining of protein from *D. biflorus* with LOX activity. The concentration of protein loaded was 2 μg/μl. (**C**) Mass spectrum of *D. biflorus* protein with LOX and lectin activity. The mass spectrum indicates the presence of two subunits of molecular mass of ~27 287 and ~28 426 Da. Inset, SDS/PAGE of purified *D. biflorus* LOX on 12 % separating and 3 % stacking gels show two closely moving bands; 20 μg of protein was loaded. (**D**) Separation of subunits of *D. biflorus* protein on a DEAE-cellulose column at 25 °C following the method described in [22]. The pooled fractions were extensively dialysed against 0.04 M Tris/HCl, pH 7.3, and 0.02 % sodium azide at 4 °C and were checked for both LOX and haemagglutination activity. Inset, SDS/PAGE pattern of the *D. biflorus* seed lectin before and after separation of subunits. Lane 1, purified lectin from *D. biflorus*; lane 2, subunit I of *D. biflorus* lectin; lane 3, subunit II of *D. biflorus* lectin; lane 4, reconstituted lectin (by mixing the two subunits in the ratio 1:1).

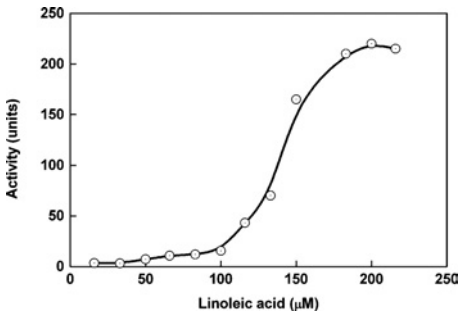

Figure 2 Substrate concentration against activity plot of *D. biflorus* LOX protein

Linoleic acid (10 mM) in alcohol was used as substrate, and the assay was performed at 25 °C.

very weak LOX activity of 8 units/mg of protein. The presence of another active LOX in *D. biflorus* [33] could contribute to the higher ratio seen in the crude extract.

The molecular masses of the subunits for purified *D. biflorus* LOX protein were analysed by MALDI–TOF. They corresponded to 27 287 and 28 426 Da (Figure 1C). SDS/PAGE analysis of the protein peak, from the DEAE-Sephacel column, revealed two close moving bands, indicating the presence of two subunits (Figure 1C, inset). The molecular masses of the subunits, as determined by SDS/PAGE, were 27 100 and 27 900 respectively.

The separated subunits (Figure 1D) were checked for LOX activity. Both the subunits had very little LOX activity (6 and 8 units/mg respectively). The SDS/PAGE pattern of the unfractionated and separated subunits is shown in Figure 1(D) (inset).

Characterization of LOX activity

The effect of varying linoleic acid concentration on LOX activity is shown in Figure 2. From the typical sigmoidal curve obtained, it is clear that an initial concentration of hydroperoxides (products of LOX activity) is necessary for activation of the enzyme. Biosynthesis of the products from $C_{18:2,n-6}$ and $C_{18:3,n-3}$ is found to proceed at a linear rate for at least 3 min, as judged from the increase in UV absorption at 234 nm. Studies have made it apparent that addition of either 1 mM of Fe^{2+} or Mn^{2+} does not stimulate enzyme activity (results not shown).

The pH optimum for LOX activity has been determined at 0.5 pH unit intervals in the range 2.5–9 using 0.1 M sodium acetate buffer (pH 2.5–5.5), 0.1 M sodium phosphate buffer (pH 6.0–8.0) and 0.1 M borate buffer (pH 8.5–9.0). The optimum pH for LOX activity was determined to be 4–5 with linoleic acid as substrate. The Michaelis constant, K_m, for linoleic acid, linolenic acid and arachidonic acid have been found to be 166, 200 and 121 μM respectively.

The kinetics of co-oxidation of β-carotene by the purified protein was followed at 460 nm (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/395/bj3950629add.htm). To characterize the co-oxidation potential of the LOX preparations, the velocity of the carotene bleaching was related to the velocity with which linoleic acid is oxidized to dienes. *D. biflorus* protein with LOX-like activity in the present study was found to be a weak co-oxidizing enzyme similar to soya beans LOX1 [34].

The reaction products of LOX activity, at pH 5.0 and 25 °C using linoleic acid as the substrate, have been analysed using SP-HPLC. The products, as a result of the activity with linoleic acid, had an absorbance maximum at 234 nm, which is indicative of the conjugated diene. Soya bean LOX1, at pH 9.0 and 25 °C, produces 13-HPOD (13-hydroperoxyoctadecadienoic acid) exclusively, while potato LOX produces 9-HPOD at pH 5.5 and 25 °C (see Supplementary Figure 2A at http://www.BiochemJ.org/bj/395/bj3950629add.htm). Product identification, carried out using SP-HPLC after reduction, reveals two peaks at 11.82 and 14.76 min. Products of LOX activity in the present study have been identified to be 13-HPOD and 9-HPOD by using soya bean LOX and potato LOX products as standards respectively. Profiles for 13-HPOD and 9-HPOD obtained are in the ratio 80:20 (see Supplementary Figure 2B).

The GC-MS pattern obtained for the sililated products of *D. biflorus* LOX protein on linoleic acid is given in Supplementary Figure 2C [13-HOD (13-hydroxyoctadecadienoic acid)]. Prominent ions were observed at *m/z* 382 (*M*), 311 [$M-71$; loss of $\cdot(CH_2)_4$-CH_3] and 227 {owing to the ions $Me_3SiO^+=CH$-$(CH=CH)_2$-$(CH_2)_4$-CH_3 in the spectrum of the derivative of 9-HOD and the ion $[(CH=CH)_2$-$CH(OSiMe_3)$-$(CH_2)_4$-$CH_3]^+$ in the spectrum of the derivative of 13-HOD}. The sililated products of soya bean LOX1 and potato LOX on linoleic acid were used as the standards. The fragmentation patterns of the sililated derivatives of 9- and 13-HOD from potato and soya bean respectively were similar (results not shown). The intensity of the *m/z* 227 ion was greater than that of the *m/z* 311 ion in the spectrum of the derivative of 9-HOD (potato LOX), whereas the reverse was true in the spectrum of the derivative of 13-HOD (soya bean LOX1; results not shown). The fragmentation pattern of peak 1 shown in the inset of Supplementary Figure 2(C) of the products in the present study (using linoleic acid as substrate) was similar to the fragmentation pattern of 13-HOD product of soya bean LOX1 with linoleic acid as the substrate (results not shown).

For steric analysis of the products, a chiral-phase column was used to identify the stereospecificity of the products. Products of the pea seed LOX1 and soya bean LOX1 (produces 98 % L-(*S*)-13-hydroperoxide product) were used as standards. The standard L-(*S*)-13-hydroperoxide product eluted at 26.412 min, while the D-(*R*)-13-HOD methyl ester eluted at 28.371 min (see Supplementary Figures 3A and 3B at http://www.BiochemJ.org/bj/395/bj3950629add.htm) [28]. In the present study, 13-HOD methyl ester of the product yielded one major peak with a retention time of 26.287 min and a minor peak at 28.113 min (see Supplementary Figure 3C).This protein exhibited similar LOX activity (on linoleic acid) comparable with pea LOX activity, with the L-(*S*)-13-HOD and D-(*R*)-13-HOD being produced in the ratio of 88:12.

Inhibition of LOX and lectin activities for detection of activity loci

The known LOX inhibitors, NDGA and ETYA, inhibited the LOX activity of the protein molecule with IC_{50} values of 161 and 101 μM respectively. The 50 % inhibition observed at 433 μM revealed that 4-nitrocatechol was a poor inhibitor (Figure 3).

Inhibition of lectin activity was carried out using different concentrations of *N*-acetylgalactosamine, a known inhibitor of *D. biflorus* lectin. Haemagglutination activity was inhibited by *N*-acetylgalactosamine with an IC_{50} value of 45 μM (results not shown).

To identify the LOX locus as being distinct from the lectin locus of the protein, *N*-acetylgalactosamine (500 μM), an inhibitor of *D. biflorus* lectin, was incubated with protein and the assay carried out. *N*-Acetylgalactosamine did not inhibit LOX activity at the concentrations used, indicating that the LOX activity locus on the molecule was different from the haemagglutination locus (results not shown). Similarly, haemagglutination was also assayed in the presence of NDGA (500 μM) and ETYA (300 μM) (results not shown). There was no effect of either NDGA or ETYA

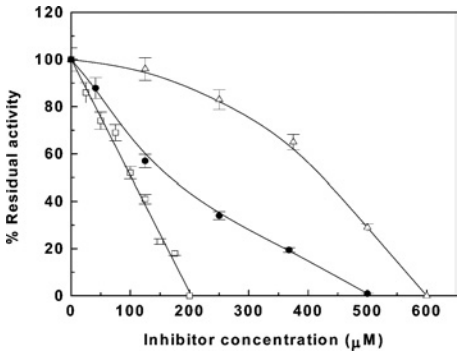

Figure 3 Effect of inhibitors on LOX activity of *D. biflorus* LOX protein

Effect of LOX inhibitors on LOX activity of purified *D. biflorus* protein. NDGA (●) and ETYA (□) stock solutions were in absolute ethanol while 4-nitrocatechol (△) was prepared in buffer. The enzyme was pre-incubated with inhibitors for 5 min. Appropriate blanks were used for the experiment. Results are an average for three experiments.

on haemagglutination, at the concentration used, indicating that the two activities were located at different loci on the molecule.

Adenine, kinetin and ANS are known to bind to the hydrophobic site of the lectin [35]. LOX activity was assayed in the presence of adenine, kinetin and ANS. All three inhibited LOX activity in a concentration-dependent manner. The IC_{50} values for LOX activity were 350 μM, 300 μM and 50 μM for adenine, kinetin and ANS respectively. ANS (50 μM) was a competitive inhibitor of LOX activity, with the K_m increasing from 161 to 333 μM; the V_{max} was not affected. However, none of the above inhibited soya bean LOX1 in the concentration range studied.

Amino acid composition, N-terminal sequence and partial sequence data

The amino acid composition of the purified *D. biflorus* LOX protein is presented in Supplementary Table 2 (at http://www.BiochemJ.org/bj/395/bj3950629add.htm). The protein was found to have very low methionine content and no cysteine. The composition matches very well with that reported for seed lectin from *D. biflorus* [22]. The N-terminal sequence of 30 amino acids and two internal peptides (~25 % of 253 amino acids) were aligned with *D. biflorus* lectin (PDB code 1LU1). The N-terminal sequence, as well as the two tryptic peptides, had 98 % similarity to the *D. biflorus* lectin [36] (Figure 4).

Prosthetic group

The purified protein, being colourless, lacked significant light absorption, even at concentrations of 1 mg/ml, between 300 and 700 nm (Figure 5A), suggesting that the enzyme lacked haem. Atomic absorption spectroscopy revealed that the protein sample contained manganese. The manganese concentration was 0.673 μg/g of protein. Iron could not be detected in the sample. We therefore concluded that the protein contains $\geqslant$0.67 μg of manganese/g of protein and no iron content. The manganese content was the same as reported for *D. biflorus* lectin [16]. However, the manganese content estimated in commercial *D. biflorus* lectin was lower (0.32 μg/g of protein). The lower LOX activity in commercial lectin could be due to the lower content of manganese.

Spectroscopic characteristics

The purified *D. biflorus* LOX protein had an absorbance maximum at 280 nm (Figure 5A). The protein was colourless and lacked significant light absorption between 300 and 600 nm. Concentrated solutions of 1 mg/ml were also colourless.

From the fluorescence spectrum for the protein obtained by excitation at 280 nm (shown in Figure 5B), the protein was found to have an emission maximum at 335 nm.

The near- and far-UV CD spectra have been monitored in the range 320–240 and 260–200 nm respectively. The near-UV CD spectrum is not well resolved even at protein concentrations of 1–1.5 mg/ml (Figure 5C). A broad peak between 250 and 290 nm has been observed. The far-UV CD spectrum reveals minima at 230 and 217 nm, establishing the predominance of β-structure. The far-UV CD spectrum for the protein is shown in Figure 5(D). The far-UV CD spectra obtained in the present study are very similar to the ones reported for the lectin from *D. biflorus* [37]. The isolated lectin also has minima at 217 and 230 nm.

Thermal stability

LOX activity of the protein was highly stable towards temperature in the range 4–95 °C. The midpoint of thermal inactivation, T_m, was found to be 85.5 °C (Figure 6A). The thermal stability of haemagglutination was determined in the range 10–75 °C. The activity was 100 % up to 75 °C (Figure 6A). However, beyond this

```
DBSL        ANIQSFSFKNFNSPSFILQGDATVSSGKLQLTKVKENGIPTPSSLGRAFYSSPIQIYDKS  60
            ANIQSFSFKNFNSPSFILQGDATVSSGKLQ----------------------------
            ********************************
                  <-------  NH2-terminal  ------->

DBSL        TGAVASWATSFTVKISAPSKASFADGIAFALVPVGSEPRRNGGYLGVFDSDVYNNSAQTV 120
            --------------------ASFADGIAFALVPVGSEPR-AGGYLGVFDSDVY-------
                                ******************  ************
                                <------------------>  <---------->
                                          T1              T2
DBSL        AVEFDTLSNSGWDPSMKHIGIDVNSIKSIATVSWDLANGENAEILITYNAATSLLVASLV 180
            ------------------------------------------------------------

DBSL        HPSRRTSYILSERVDITNELPEYVSVGFSATTGLSEGYIETHDVLSWSFASKLPDDSTAE 240
            ------------------------------------------------------------

DBSL        PLDLASYLVRNVL 253
            -------------
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Figure 4 Comparison of the N-terminal sequence and internal peptide sequences of *D. biflorus* LOX protein with the reported sequence of *D. biflorus* seed lectin [36]

The sequence alignment was carried out by Clustal W version 1.82. T1 and T2 are peptides of Tos-Phe-CH_2Cl–trypsin digest sequence obtained in the present study. DBSL, *D. biflorus* seed lectin.

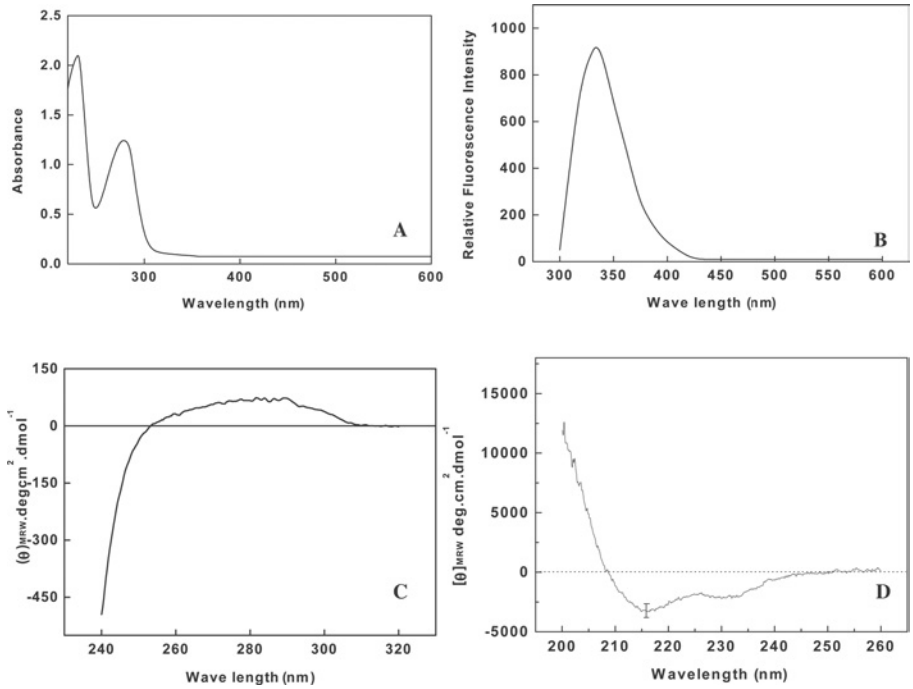

Figure 5 Spectroscopic analysis of *D. biflorus* LOX protein

(**A**) UV–visible spectra of purified *D. biflorus* LOX. The absorption spectrum was recorded from 220 to 600 nm using a 1 cm pathlength cell. The protein was taken in 20 mM sodium phosphate buffer, pH 6.0. (**B**) Fluorescence spectrum of *D. biflorus* LOX. The bandwidths for excitation and emission monochromators were both 5 nm. The emission spectrum of the protein was recorded in the region of 300–400 nm after excitation at 280 nm. The protein concentration used was 50 mg/ml. Appropriate buffers were used for correcting the fluorescence intensity of the solvent. (**C**) Near-UV CD spectrum for *D. biflorus* LOX. The pathlength of the cell used was 1 cm in the near-UV region of 240–320 nm. A protein concentration of 1–1.5 mg/ml was used. (**D**) Far-UV CD spectrum for *D. biflorus* LOX. The protein concentration used was 0.42 mg/ml in the far-UV region (200–260 nm) with a cell pathlength of 1 mm. Measurements were made at 25 °C at a scan speed of 10 nm/min, and the spectrum represented is an average for at least three accumulations.

temperature, aggregation of protein because of high concentrations rendered it difficult to test activity. The molar ellipticity of the protein at 217 nm increased as a function of temperature. There was 47.6 % increase in the ellipticity value up to 90 °C (Figure 6A).

The Arrhenius plots (Figure 6B) of inverse temperature against the logarithm of velocity were constructed for the activity in the range 78–95 °C for the calculation of inactivation parameters. The reaction rates were estimated by UV analysis at 234 nm. The E_a, ΔG^*, ΔH^* and ΔS^* were found to be 49.7 kcal $\cdot$ mol^{-1} (1 cal = 4.184 J), 25.8 kcal $\cdot$ mol^{-1}, 49 kcal $\cdot$ mol^{-1} and 66.1 cal $\cdot$ mol^{-1} $\cdot$ K^{-1} at 78 °C respectively.

The LOX characteristics for the purified protein are summarized in Table 1.

Polyclonal antibodies raised against soya bean LOX1

Rabbit antisera were prepared against soya bean LOX1 and were checked for cross-reactivity with *D. biflorus* purified protein with LOX activity; there was no cross-reaction. Furthermore, the antisera did not cross-react with commercial *D. biflorus* lectin. There is no similarity in the lectin sequence compared with reported plant LOX sequences.

DISCUSSION

The LOX activity from *D. biflorus* is 1800 units/g of seed (Supplementary Table 1). This is probably the richest source of LOX activity from plants. Since structural studies of the purified molecule have suggested similarities with lectin profile, we looked at lectin LOX activity during purification to ascertain the association of LOX activity with the lectin molecule, which remained constant, suggesting that the two activities are associated with the same molecule. The homogenous protein reported in the present paper was found to have a haemagglutination of 45 $\pm$ 5 units/mg of protein (see the Materials and methods section), which is comparable with that of the commercial lectin from *D. biflorus*. The protein has a specific activity for LOX of 154 units/mg (Supplementary Table 1) and a pH optimum of 4.0–5.0. This is one of the highest specific activities associated with an acidic LOX from a leguminous family.

The protein had a molecular mass of $\sim$ 110 000 Da (Figure 1A) with two sets of subunits of very close mobility. The molecular masses of subunits determined by SDS/PAGE were 27 100 and 27 900 Da respectively. The corresponding molecular masses of the subunits by MALDI–TOF were 27 287 and 28 426 Da. The molecular mass and amino acid composition of the protein was very similar to seed lectin from *D. biflorus* [22,36]. Total carbohydrate content [38] in the purified protein was found to be 1.3 %, which is exactly the same as reported for the *D. biflorus* lectin [16]. Protein from the present study and commercial lectin from *D. biflorus* were stained for glycosylation [39]. Both proteins were glycosylated, as revealed by the pink bands seen after periodic acid staining (results not shown).

The N-terminal sequence of both subunits was identical. Tryptic peptides of subunit I were separated and subjected to amino acid sequencing. Both the N-terminal sequence (30 residues) and two internal peptide sequences were aligned with *D. biflorus* seed lectin reported in literature (PDB code 1LU1) (Figure 4). The above results further corroborated the identification of the protein

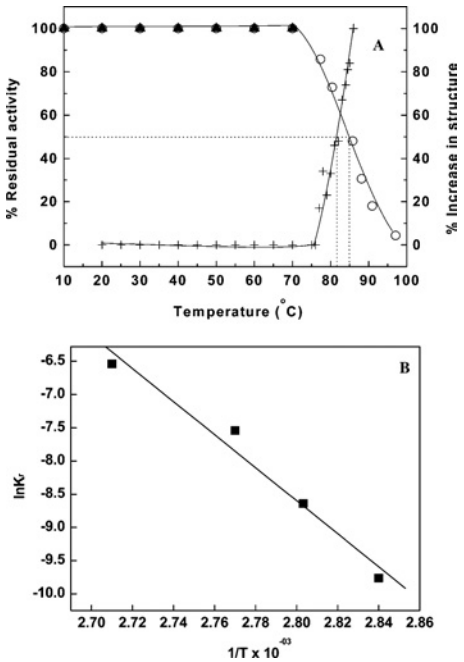

Figure 6 Thermal stability of *D. biflorus* LOX protein

(**A**) Kinetics of thermal inactivation. ○, *D. biflorus* LOX; ▲, commercial lectin. Samples were incubated at the required temperature in the range 10–95 °C in 20 mM sodium phosphate buffer, pH 6.0. Aliquots of the enzyme were drawn at different time intervals, cooled to 4 °C and assayed for residual activity. Commercial lectin aggregated at temperatures above 75 °C, making it difficult to test activity. +, Purified protein in 20 mM sodium phosphate buffer, pH 6.0, was used to follow the changes in structure at 217 nm using a 1 mm cell in the temperature range 25–85 °C. Temperature increase at a rate of 1 °C/min was achieved using a Peltier attachment. The mean residue mass of 115 was used to calculate the molar ellipticity. The absolute increase in ellipticity at 217 nm at 90 °C was taken as 100 % increase, and the relative increase in ellipticity was calculated for different temperatures. (**B**) Arrhenius plot for *D. biflorus* LOX.

Table 1 LOX characteristics of purified *D. biflorus* protein

Value	Parameter
Molecular mass (Da)/structure	~110000/tetramer
pH optimum	4.0–5.0
K_m (μM)	
Linoleic acid	166
Linolenic acid	200
Arachidonic acid	121
V_{max} (μmol/min)	
Linoleic acid	66.6
Linolenic acid	55
Arachidonic acid	100
IC_{50} (μM)	
NDGA	161
4-Nitrocatechol	433
ETYA	101
ANS	50
Kinetin	300
Adenine	350
Structure	Predominantly β-sheet
Product ratio with linoleic acid (9-HPOD/13-HPOD)	20:80
T_m (°C)	85.5
Thermal inactivation parameters at 78 °C	
E_a (kcal · mol^{-1})	49.7
ΔG* (kcal · mol^{-1})	25.8
ΔH* (kcal · mol^{-1})	49
ΔS* (cal · mol^{-1} · K^{-1})	66.1

as a lectin. Subunit II (241 amino acid residues) is reported to be post-translationally formed from subunit I (253 amino acid residues) by the removal of 12 residues from its C-terminus [36].

The curves of substrate concentration against reaction rate had a sigmoidal shape, indicating that the LOX activity is very similar to soya bean LOX 1 with the requirement of hydroperoxides for activation of the molecule (Figure 2). The reported K_m for various substrates, given in Table 1, indicated that this molecule has a good ability to convert PUFAs into their respective peroxides. The classical inhibitors of LOXs, such as ETYA (substrate analogue) and NDGA (free radical scavenger), inhibited the LOX activity associated with the molecule with IC_{50} values of 101 and 161 μM respectively (Table 1). 4-Nitrocatechol (binds to iron–enzyme complex) did not inhibit the activity. The protein had characteristics similar to other LOXs for co-oxidation of β-carotene (see Supplementary Figure 1) [34].

13-HPOD and 9-HPOD are the major products of the LOX reaction (see Supplementary Figure 2). Both the stereochemistry of the hydroperoxides and the nature of insertion of oxygen across the double bond to produce either 9- or 13-HPOD indicate that the lectin molecule behaves like the classical LOX molecule [3]. The functional diversity of LOX isoforms enable the plant to respond appropriately to environmental challenges [5]. LOX products (9*S*)-hydroperoxy and (13*S*)-hydroperoxy derivatives of PUFAs are considered to be of central importance for the production of a plethora of oxylipins found in plants. Jasmonates (signalling compounds), antimicrobial and antifungal compounds (leaf aldehydes or divinyl ethers) and volatiles are among the numerous products [5]. Jasmonates and their octadecanoid precursors are reported to act as a master switch in plant development and stress adaptation.

Haemagglutination, measured in the presence of the inhibitors of LOX, and LOX activity measured in the presence of *D. biflorus* lectin inhibitor, suggest that the location of LOX activity is away from the haemagglutination activity on the protein molecule. The separated subunits of the protein individually do not have either haemagglutination or LOX activity. Efforts to reconstitute the molecule with equimolar concentrations of subunits have not been successful.

The CD spectra of the molecule in the near-UV and far-UV region are found to have the characteristic features of β-structure with minima at 217 nm. Near-UV CD band is very broad in the region 250–290 nm and not well resolved even at high concentrations of protein (1–1.5 mg/ml). Lectin from *D. biflorus* has two well-resolved peaks in the near-UV regions at 265 and 290 nm [37].

The majority of the plant LOXs are reported to have low thermal stability. The T_m for LOX activity associated with protein in the present study was found to be 85.5 °C, suggesting that the molecule could withstand high temperatures. The thermal inactivation mechanism was found to follow typical first-order reaction kinetics. The corresponding E_a, ΔH^*, ΔG^* and ΔS^* values are given in Table 1. LOX activity is highly thermostable with a ΔH^* value of 48.99 kcal · mol^{-1} compared with 9.9 kcal · mol^{-1} for soya bean LOX1. Inactivation of both LOX and haemagglutination is found to follow the same pattern (Figure 6A).

The rabbit antiserum raised against soya bean LOX1 did not cross-react either with the protein of the present study or with the commercial *D. biflorus* lectin. Sequence alignment between the *D. biflorus* lectin and LOXs reported from plant sources did not reveal any significant homology (results not shown).

The protein in the present study has manganese as the prosthetic group. Fe^{2+} and Mn^{2+} are metal ions reported from LOXs from different sources [3,11]. The lectin from *D. biflorus* is reported to

contain Mn^{2+} [16]. The presence of Mn^{2+} in the lectin molecule may allow it to function as a LOX molecule, very similar to other manganese LOXs [40]. Lectin from *D. biflorus* prepared by the method reported previously [16] is found to possess a lectin activity of 45 units/mg and LOX activity of 150 units/mg, against corresponding values of 45 ± 5 units/mg and 8 units/mg respectively for commercial lectin. The manganese content of commercial lectin is 0.32 μg/g of protein. The manganese content may have a bearing on LOX activity. One of the unique features of legume lectins is their variable quaternary structure [35]. They can associate into tetramers or dimers. This unique feature of lectin from *D. biflorus* [35] could explain its ability to bind hydrophobic molecules. Some of the legume lectins contain multiple hydrophobic sites which bind the fluorescent probes ANS and 2,6-toluidinylnaphthalenesulphonate with varying affinities, typically in the range 10^3–10^4 M^{-1} [41]. The seed lectin of *D. biflorus* contains two identical sites for binding adenine, with affinities ranging from 2×10^5 to 5×10^4 M^{-1} [42]. Cytokinins, plant growth regulators, are reported to compete for the same site with similar affinities [43]. All the three inhibited LOX activity of seed lectin in a concentration-dependent manner and the nature of inhibition with ANS was competitive. Hence, the substrate, linoleic acid, must be approaching manganese through the hydrophobic site of the lectin molecule. Lectin has two carbohydrate-binding sites per tetramer. There is no interaction between the carbohydrate-binding and adenine-binding sites [35].

There is a need to develop appropriate strategies for deployment of transgenics for pest management. Thus the seed lectin molecule of *D. biflorus* with its associated LOX activity can lead to a mechanism for imparting/improving pest resistance in other plant crops.

Conclusion

Plant lectins have reported applications in medicine, industry and research [44]. The function of lectins in plants remains a mystery. They play an important role in (i) seed maturation, and (ii) cell wall assembly, defence mechanisms or rhizobial nodulation of legume roots. The hydrophobic site on the tetrameric lectin from *D. biflorus* along with an Mn^{2+} ion accounts for the observed LOX activity of seed lectin. There is no interaction between the lectin and LOX locus. LOX activity associated with this molecule adds a new dimension to our understanding of lectin functions.

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REFERENCES

1 Ranjekar, P. K., Patankar, A., Gupta, V., Bhatnagar, R., Bentur, J. and Kumar, P. A. (2003) Genetic engineering of crop plants for insect resistance. Curr. Sci. **84**, 321–329
2 Ryan, C. A. (1990) Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu. Rev. Phytopathol. **28**, 239–249
3 Brash, A. R. (1999) Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. J. Biol. Chem. **274**, 23679–23682
4 Siedow, J. N. (1991) Plant lipoxygenase: structure and function. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42**, 145–188
5 Feussner, I. and Wasternack, C. (2002) The lipoxygenase pathway. Annu. Rev. Plant Biol. **53**, 275–297
6 Porta, H. and Rocha-Sosa, M. (2002) Plant lipoxygenases: physiological and molecular features. Plant Physiol. **130**, 15–21
7 Gardner, H. W. (1991) Recent investigations into the lipoxygenase pathway of plants. Biochem. Biophys. Acta **1084**, 221–239
8 Maccarone, M., Melino, G. and Finazzi-Agro, A. (2001) Lipoxygenase and their involvement in programmed cell death. Cell Death Differ. **8**, 776–784
9 Nelson, M. J. and Seitz, S. P. (1994) The structure and function of lipoxygenase. Curr. Opin. Struct. Biol. **4**, 878–884
10 Brash, A. R., Boeglin, W. E., Chang, M. S. and Shieh, B. H (1996) Purification and molecular cloning of an 8*R*-lipoxygenase from the coral *Plexaura homomalla* reveal the related primary structure of *R*- and *S*-lipoxygenases. J. Biol. Chem. **271**, 20949–20957
11 Su, C. and Oliw, E. H. (1998) Manganese lipoxygenase: purification and characterization. J. Biol. Chem. **273**, 13072–13079
12 Talbot, C. F. and Etzler, M. E. (1978) Development and distribution of *Dolichos biflorus* lectin as measured by radioimmuno assay. Plant Physiol. **61**, 847–850
13 Hamelryck, T. W., Remy, L., Bouckaert, J. and Wyns, L. (1998) Structural features of the legume lectins. Trends Glycosci. Glycotechnol. **10**, 349–360
14 Jeffrey, C. J. (1999) Moonlighting proteins. Trends Biochem. Sci. **24**, 8–11
15 Sudharshan, E. and Rao, A. G. A. (1999) Involvement of cysteine residues and domain interactions in the reversible unfolding of lipoxygenase-1. J. Biol. Chem. **274**, 35351–35358
16 Kocourek, J., Jamieson, G. A., Votruba, T. and Hořejší, V. (1977) Studies on phytohaemagglutinins. 1. Some properties of the lectins of horse gram seeds (*Dolichos biflorus* L.). Biochim. Biophys. Acta **500**, 344–360
17 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
18 Guss, P. L., Richardson, T. and Stahmann, M. A. (1967) The oxidation–reduction enzymes of wheat. III. Isoenzymes of lipoxidase in wheat fractions and soybean. Cereal Chem. **44**, 607–609
19 Shimizu, T., Honda, Z. I., Miki, I., Seyama, Y., Izumi, T., Rådmark, O. and Samuelsson, B. (1990) Potato arachidonte 5-lipoxygenase: purification, characterization, and preparation of 5(*S*)-hydroperoxyeicosatetranoic acid. Methods Enzymol. **187**, 296–307
20 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275
21 Kumar, N. S. and Rao, D. R. (1986) The nature of lectins from *Dolichos lablab*. J. Biosci. **10**, 95–109
22 Carter, W. G. and Etzler, M. E. (1975) Isolation and characterization of subunits from the predominant form of *Dolichos biflorus* lectin. Biochemistry **14**, 2685–2689
23 Sanz, A., Perz, A. G. and Olias, J. M. (1994) Pigment cooxidation activity by chickpea lipoxygenases. Food Chem. **50**, 231–235
24 Axelrod, B., Cheesbrough, T. M. and Laakso, S. (1981) Lipoxygenase from soybeans. Methods Enzymol. **71**, 441–451
25 Feussner, I. and Kühn, H. (1995) The lipid body lipoxygenase from cucumber seedlings exhibits unusual reaction specificity. FEBS Lett. **367**, 12–14
26 Mulliez, E., Leblanc, J.-P., Girerd, J.-J., Rigaud, M. and Chottard, J.-C. (1987) Lipoxygenase from potato tubers: improved purification and physicochemical characteristics. Biochem. Biophys. Acta **916**, 13–17
27 Sudharshan, E. and Rao, A. G. A. (1997) Rapid method to separate the domains of soybean lipoxygenase-1: identification of the interdomain interactions. FEBS Lett. **406**, 184–188
28 Kühn, H., Wiesner, R., Lankin, V. Z., Nekrasov, A., Alder, L. and Schewe, T. (1987) Analysis of the stereochemistry of lipoxygenase-derived hydroxypolyenoic fatty acids by means of chiral phase high-pressure liquid chromatography. Anal. Biochem. **160**, 24–34
29 Reynolds, P. A. and Barbara, P. K. (1982) Characterization of a type-1 lipoxygenase from peas. J. Agric. Food Chem. **30**, 1157–1163
30 Hamberg, M., Gerwick, W. H. and Åsen, P. A. (1992) Linoleic acid metabolism in the red alga *Lithothamnion coralloides*: biosynthesis of 11(*R*)-hydroxy-9(*Z*),12(*Z*)-octadecadienoic acid. Lipids **27**, 487–493
31 Bidlingmeyer, B. A., Cohen, S. A. and Tarvin, T. L. (1984) Rapid analysis of amino acids using pre-column derivatization. J. Chromatogr. **336**, 93–104
32 Freund, J. (1947) Some aspects of active immunization. Annu. Rev. Microbiol. **1**, 291–308
33 Roopashree, S. and Rao, A. G. A. (2004) Thermostable iron containing low molecular weight lipoxygenase from *Dolichos biflorus*: purification and characterization. FASEB J. **18**, C13
34 Grosch, W., Laskawy, G. and Weber, F. (1976) Formation of volatile carbonyl compounds and cooxidation of β-carotene by lipoxygenase from wheat, potato, flax and beans. J. Agric. Food Chem. **24**, 456–459
35 Hamelryck, T. W., Loris, R., Boukaert, J., Dao-Thi, M.-H., Strecker, G., Imberty, A., Fernandez, E., Wyns, L. and Etzler, M. E. (1999) Carbohydrate binding, quaternary structure and a novel hydrophobic binding site in two legume lectin oligomers from *Dolichos biflorus*. J. Mol. Biol. **286**, 1161–1177
36 Schnell, D. J. and Etzler, M. E. (1987) Primary structure of the *Dolichos biflorus* seed lectin. J. Biol. Chem. **262**, 7220–7225

37 Père, M., Bourrillon, R. and Jirgensons, B. (1975) Circular dichroism and conformational transition of *Dolichos biflorus* and *Robinia pseudoacacia* lectins. Biochim. Biophys. Acta **393**, 31–36
38 Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**, 350–356
39 Zacharius, R. M., Zell, T. E., Morrison, J. H. and Woodlock, J. J. (1969) Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. **30**, 148–152
40 Su, C., Sahlin, M. and Oliw, E. (2000) Kinetics of manganese lipoxygenase with a catalytic mononuclear redox center. J. Biol. Chem. **275**, 18830–18835
41 Roberts, D. D. and Goldstein, I. J. (1983) Binding of hydrophobic ligands to plant lectins: titration with arylaminonaphthalenesulfonates. Arch. Biochem. Biophys. **224**, 479–484
42 Gegg, C. V., Roberts, D. D., Segel, I. H. and Etzler, M. E. (1992) Characterization of the adenine binding sites of two *Dolichos biflorus* lectins. Biochemistry **31**, 6938–6942
43 Mok, M. C., Mok, D. W. S. and Armstrong, D. J. (1978) Differential cytokinin structure–activity relationship in *Phaseolus*. Plant Physiol. **61**, 72–75
44 Lis, H. and Sharon, N. (1986) Properties, functions and applications in biology and medicine. In The Lectins (Liener, I. E., Sharon, N. and Goldstein, I. J., eds.), pp. 293–370, Academic Press, New York

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